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# *Geopora ahmadii* sp. nov. from Pakistan

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Abstract—A new species, *Geopora ahmadii,* is described and illustrated based on material from Punjab, Pakistan. This species is characterized by sessile, cup- to saucer-shaped, partly immersed apothecia with whitish to grayish hymenial surfaces; broad ellipsoid, mostly uniguttulate ascospores; and brown excipular hairs. Phylogenetic analyses of the nrDNA ITS region with maximum parsimony, maximum likelihood, and Bayesian inference methods reveal that *G. ahmadii* is distinct from other described *Geopora* species. A collection previously identified as *Geopora arenosa* from Rawalakot, Pakistan, likely represents a second locality of *G. ahmadii*.

Key words—*Ascomycota,* hypogeous fungi, *Pezizales, Pyronemataceae,* taxonomy

## Introduction

*Geopora* Harkn. (*Pyronemataceae*, *Pezizales*) is characterized by (1) ascomata that occur entirely or partially below ground and are covered with brown, septate excipular hairs; (2) a whitish, grayish, or yellowish-gray hymenium; and (3) smooth, mostly uniguttulate ascospores. Because ascomata appear infrequently and are hypogeous at some developmental

stage, *Geopora* specimens are infrequently collected. Species delimitation is additionally challenging due to the scarcity of distinctive morphological characters, with measurement ranges overlapping among species (Tamm & al. 2010, Guevara-Guerrero & al. 2012, Flores-Rentería & al. 2014).

Identification of *Geopora* species has relied primarily on ascospore shape and size, position of apothecia in the ground, and the length of excipular hairs (Burdsall 1965, 1968; Tamm & al. 2010; Flores-Rentería & al. 2014). Molecular analyses by Tamm & al. (2010) showed that well supported clades are not congruent with morphological species concepts. The combination of molecular and morphological data is considered the most reliable approach to define species in this genus (Southworth & Frank 2011, Guevara-Guerrero & al. 2012, Flores-Rentería & al. 2014).

Perry & al. (2007) studied the phylogenetic relationships of *Pyronemataceae*. Using LSU ribosomal DNA (rDNA) sequence data, they suggested that *Geopora* is monophyletic. Hansen & al. (2013) determined that *Geopora* was sister to *Tricharina* in the larger *Scutellinia–Trichophaea* lineage. Neither study included species representing *Phaeangium* Pat. or *Picoa* Vittad. Stielow & al. (2013) suggested that *Geopora* was paraphyletic including species of both *Phaeangium* and *Picoa*. They also found that *Geopora pellita* (Sacc.) T. Schumach. was phylogenetically isolated from other *Geopora* species and created the new genus *Hoffmannoscypha* Stielow & al. to accommodate this taxon.

During our studies of ectomycorrhizal fungi, we found *Geopora* specimens growing in groups on damp soil in Punjab, Pakistan. Molecular phylogenetic analysis of the ITS rDNA region combined with morphological evaluation support the recognition of our collections as a new species. This species is described, illustrated, and compared with other *Geopora* species.

## Materials & methods

### Morphological studies

Ascomata were collected and dried in a food dehydrator at 39 °C for 7–9 hours. Shape, texture, and dimensions of important characters were recorded from fresh ascomata. Colors were compared to the Munsell Soil Color Charts (1975). Dried voucher specimens are deposited at the University of the Punjab Herbarium, Lahore, Pakistan (LAH) and the Farlow Herbarium, Harvard University, Cambridge, MA, USA (FH).

Sections of specimens were mounted in water and Congo red in ammonia (0.3% in commercial ammonia cleaner) to increase contrast for microscopic observations.

Micromorphological analysis, photographs, and measurements were made

using an Olympus Bx40 light microscope with Olympus XC50 digital camera and Microsuite Special Edition software 3.1. Sections were made using a freezing microtome. Measurements include the typical range with extremes given in parentheses. Q values (length/width ratios) are given for ascospores.

#### DNA extraction, PCR amplification, DNA sequencing

Genomic DNA was extracted from a small piece of an ascoma by a modified CTAB method (Gardes & Bruns 1993). The internal transcribed spacer region (ITS1+5.8S+ITS2) of the nuclear ribosomal RNA gene was amplified using the primer pair ITS1F and ITS4 (White & al. 1990, Gardes & Bruns 1993) and the RED Extract-N-Amp PCR ReadyMix. PCR cycling parameters comprised initial denaturation (94° C for 1 min), 35 cycles (94 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min), and final extension 72 °C (8 min). Amplified PCR products were outsourced to Macrogen, (Seoul, Republic of Korea) for purification and bidirectional sequencing.

#### Sequence alignment & phylogenetic analyses

*Geopora* sequences downloaded from GenBank included those studied by Tamm & al. (2010) and sequences representing recently described species—*G. cercocarpi* D. Southw. & J.L. Frank, *G. gilkeyae* (Burds.) G. Guevara & al., *G. pinyonis* Flores-Rent. & Gehring, and *G. tolucana* G. Guevara & al. (Flores-Rentería & al. 2014, Guevara-Guerrero & al. 2012, Southworth & Frank 2011). *Tarzetta catinus* (Holmsk.) Korf & J.K. Rogers and *Trichophaea hybrida* (Sowerby) T. Schumach. (*Pyronemataceae*, *Pezizales*) were selected as the outgroup because they are closely related to *Geopora* (Perry & al. 2007).

Manually edited sequences were assembled in BioEdit v7.2.6 (www.mbio.ncsu.edu/bioedit/bioedit.html). All sequences were trimmed with the conserved motifs 5′-(…GAT)CATTA– and –GACCT(CAAA…)-3′ (Dentinger & al. 2011), and the alignment portions between them were included in the analysis. Sequences retrieved from NCBI GenBank were aligned by Muscle v3.7 (Edgar 2004) with default parameters using Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura & al. 2011).

Maximum parsimony (MP) analysis was performed with PAUP 4.0b on XSEDE (Swofford 1991), available on the Cipres Gateway v3.3 (Miller & al. 2010). All characters were equally weighted and gaps were treated as missing data. The heuristic search option with tree-bisection-reconnection (TBR) branch swapping and 1000 random sequence additions were used to infer trees. Clade robustness was assessed using a bootstrap analysis with 500 replicates (Felsenstein 1985). A maximum likelihood (ML) analysis was carried out with RAxML XSEDE on the Cipres Gateway, using the general time-reversible (GTR) model of nucleotide substitution (Stamatakis & al. 2008). Nodal support was determined from 1000 bootstrap replicates.

Bayesian analysis was done with a Markov chain Monte Carlo (MCMC) coalescent approach implemented in Beast v1.8.2 (Drummond & Rambaut 2007),

Table 1. *Geopora* isolates and outgroup included in phylogenetic analyses [Clade designations sensu Tamm & al. 2010]

| Original ID | Clade | Country | Voucher | GenBank | Reference |
|---|---|---|---|---|---|
| *Tarzetta catinus* | | Estonia | TAAM 192291 | FM206478 | Tamm & al. 2010 |
| *Trichophaea hybrida* | | Estonia | TAAM 192334 | FM206477 | Tamm & al. 2010 |
| *G. cercocarpi* | | USA, OR | SOC 1590 | HQ283090 | Southworth & Frank 2011 |
| *G. cercocarpi* | | USA, OR | SOC 1590 | NR121491 | Southworth & Frank 2011 |
| *G. pinyonis* | | USA, AR | DGB 27586 | KF768653 | Flores-Rentería & al. 2014 |
| *G. pinyonis* | | USA, AR | DGB 27586 | KF768652 | Flores-Rentería & al. 2014 |
| *G. tolucana* | | Mexico | ITCV 1081 | HQ184961 | Guevara-Guerrero & al. 2012 |
| *G. tolucana* | | Mexico | ITCV 1081 | HQ184960 | Guevara-Guerrero & al. 2012 |
| ***G. ahmadii*** | **IX** | **Pakistan** | **MSM#0091 [T]** | **KY805995** | **This paper** |
| ***G. ahmadii*** | **IX** | **Pakistan** | **MSM#00163** | **KY805996** | **This paper** |
| *G. arenicola* | IX | Estonia | TAAM 192329 | FM206473 | Tamm & al. 2010 |
| *G.* sp. | IX | Estonia | TAAM 192324 | FM206471 | Tamm & al. 2010 |
| *G. arenicola* | IX | Estonia | TAAM 192330 | FM206472 | Tamm & al. 2010 |
| *G. foliacea* | IX | Estonia | TAAM 192323 | FM206470 | Tamm & al. 2010 |
| Ectomycorrhizal | X | France | ECM 2 | AJ410862 | El Karkouri & al. 2004 |
| Ectomycorrhizal | X | France | ECM 95 | AJ410865 | El Karkouri & al. 2004 |
| Ectomycorrhizal uncultured | X | Spain | Riv-4 | EF484934 | Rincón & al. 2007 |
| *G.* cf. *sepulta* | X | Estonia | TAAM 113526 | FM206476 | Tamm & al. 2010 |
| *G. cooperi* | X | | 101GA | AF387651 | Gutierrez & al. (unpubl.) |
| *G. cooperi* | X | | 108GC | AF387649 | Gutierrez & al. (unpubl.) |
| *G. cooperi* | X | | 109GC | AF387650 | Gutierrez & al. (unpubl.) |
| *G. gilkeyae* | | USA, CA | src515 | DQ974731 | Smith & al. 2007 |
| *G. arenicola* | VIII | Estonia | TAAM 188666 | FM206449 | Tamm & al. 2010 |
| *G. arenicola* | VIII | Estonia | TAAM 188339 | FM206446 | Tamm & al. 2010 |
| *G. arenicola* | VIII | Estonia | TAAM 117708 | FM206460 | Tamm & al. 2010 |
| *G. arenicola* | VIII | Estonia | TAAM 188293 | FM206440 | Tamm & al. 2010 |
| *G. arenicola* | VIII | Estonia | TAAM 188292 | FM206439 | Tamm & al. 2010 |
| *G. arenicola* | VIII | Estonia | TAAM 135060 | FM206433 | Tamm & al. 2010 |
| *G. arenicola* | VIII | Estonia | TAAM 116784 | FM206462 | Tamm & al. 2010 |
| *G. sepulta* | VII | Estonia | TAAM 192311 | FM206432 | Tamm & al. 2010 |

| Original ID | Clade | Country | Voucher | GenBank | Reference |
|---|---|---|---|---|---|
| *G. sepulta* | VII | Estonia | TAAM 192333 | FM206431 | Tamm & al. 2010 |
| *G. foliacea* | V | Finland | H RS-34685 | FM206428 | Tamm & al. 2010 |
| *G. foliacea* | V | Finland | H RS-29584 | FM206424 | Tamm & al. 2010 |
| *G. cervina* | V | Finland | H RS-17984 | FM206426 | Tamm & al. 2010 |
| *G. cervina* | VI | Estonia | TAAM 192232 | FM206420 | Tamm & al. 2010 |
| *G. tenuis* | VI | Estonia | TAAM 192302 | FM206429 | Tamm & al. 2010 |
| *G.* sp. | VI | Tajikistan | TAAM 116668 | FM206475 | Tamm & al. 2010 |
| *G. tenuis* | IV | Estonia | TAAM 188326 | FM206397 | Tamm & al. 2010 |
| *G. tenuis* | IV | Finland | H RS-09584 | FM206402 | Tamm & al. 2010 |
| *G. tenuis* | IV | Estonia | TAAM 188331 | FM206396 | Tamm & al. 2010 |
| *G. cervina* | IV | Estonia | TAAM 192293 | FM206401 | Tamm & al. 2010 |
| *G. cervina* | III | Estonia | TAAM 117479 | FM206413 | Tamm & al. 2010 |
| *G. cervina* | III | Finland | H RS-07186 | FM206406 | Tamm & al. 2010 |
| *G. cervina* | III | Estonia | TAAM 117884 | FM206409 | Tamm & al. 2010 |
| *G. cervina* | III | Estonia | TAAM 192321 | FM206410 | Tamm & al. 2010 |
| *G. arenicola* | III | Estonia | TAAM 117952 | FM206412 | Tamm & al. 2010 |
| *G. cervina* | II | Estonia | TAAM 188304 | FM206417 | Tamm & al. 2010 |
| *G. cervina* | II | Estonia | TAAM 117898 | FM206419 | Tamm & al. 2010 |
| *G. arenicola* | II | Estonia | TAAM 188517 | FM206418 | Tamm & al. 2010 |
| *G. cervina* | I | Finland | H RS-06986 | FM206387 | Tamm & al. 2010 |
| *G. cervina* | I | Estonia | TAAM 188655 | FM206390 | Tamm & al. 2010 |
| *G. cervina* | I | Estonia | TAAM 117854 | FM206391 | Tamm & al. 2010 |
| *G. cervina* | I | Estonia | TAAM 117659 | FM206389 | Tamm & al. 2010 |

with an uncorrelated lognormal relaxed clock for rate variation across the tree. A Bayesian skyride coalescent tree GMRF prior with the GTR+I+G model of nucleotide substitution was used in all simulations, with a randomly generated starting tree. Four independent runs of 10 million generations were undertaken. Tracer v1.6.0 (Drummond & Rambaut 2007) was used to check the effective sample size (ESS), and burn-in values were adjusted to achieve a net ESS of at least 200. Upon removal of a portion of each run as burn-in, log files and trees files were combined in LogCombiner v.1.8.2. Finally, a consensus tree (0% burn-in) was generated using TreeAnnotator v1.8.2 and visualized in FigTree v1.4.2.

Sequences of *Geopora ahmadii* generated during this study were submitted to GenBank. Accession numbers for the sequences downloaded from GenBank and those sequenced during this study are given in Table 1.

## Taxonomy

***Geopora ahmadii*** Saba, T. Ashraf, Khalid & Pfister, **sp. nov.** Figs 1, 2

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Differs from *Geopora arenicola* by its partly immersed apothecia that are larger in diameter, cupulate when young but saucer-shaped when older, by its broadly ellipsoid ascospores, and by its ITS sequence with 49–52 autapomorphies.

Type: Pakistan, Punjab, Lahore, University of the Punjab, Department of Botany, Botanical Garden, 31°29′56″N 74°17′57″E, 6 March 2009, leg. M. Saba, T. Ashraf & A.N. Khalid, MSM#0091 (**Holotype**, LAH 310019; GenBank KY805995).

Etymology: Named in honor of Dr. Sultan Ahmad (1910–1983), eminent pioneering mycologist in Pakistan.

Apothecia partly immersed in soil, sessile, fleshy, cup-shaped at early stages and saucer-shaped at older stages when becoming thin, flat-discoid, ≤15–25 mm in diameter and 4 mm deep when fresh; when dry shrinking to 6–10 mm diam; disc grey and smooth when fresh, whitish beige to whitish grey when

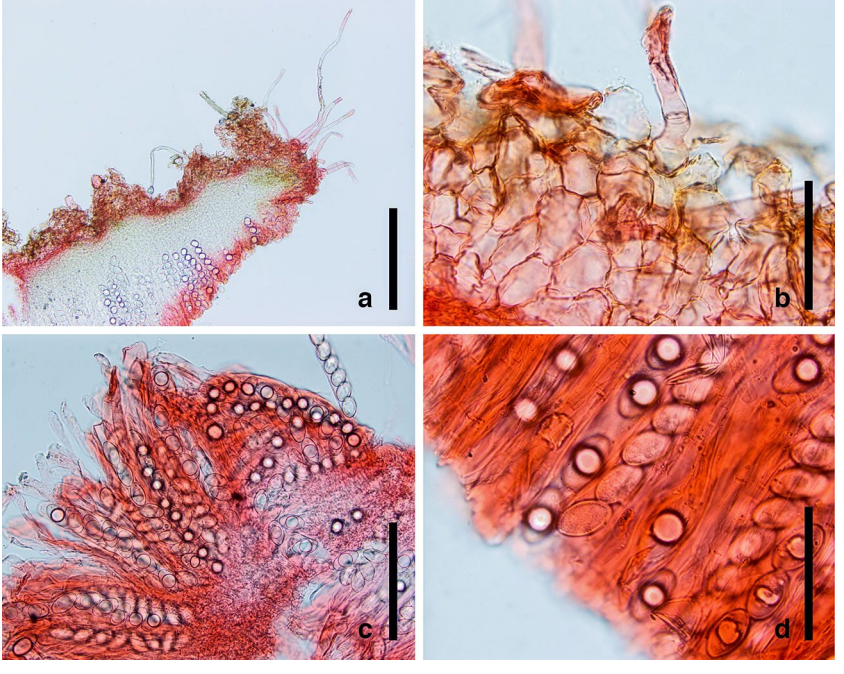


Fig. 1. *Geopora ahmadii* (holotype, LAH310019). a. Section of ascoma showing hymenium, subhymenium, excipulum, and excipular hairs; b. Excipular cells with single excipular hair; c. Asci and paraphyses; d. Asci and paraphyses, with detail of an ascospore with a single guttule (insert). Scale bars: a = 200 µm; b, d = 50 µm; c = 100 µm.

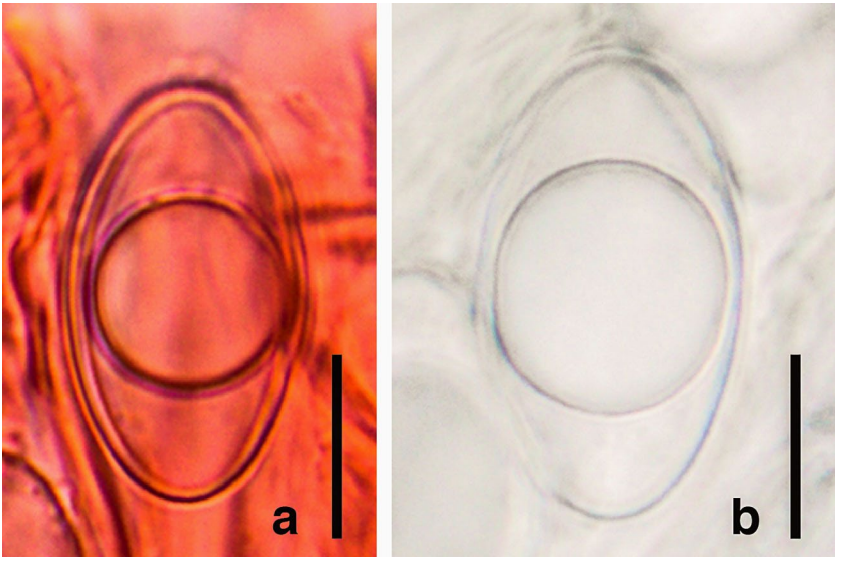


Fig. 2. *Geopora ahmadii* (holotype, LAH310019). Ascospores, each with a single guttule: a. Mounted in Congo red in ammonia; b. Mounted in water. Scale bars = 10 µm.

rehydrated; receptacle dark brown, warted and hairy. Margin wavy at young stages, splitting into 5–6 lobes at older stages.

Hymenium 180–220 µm thick. Asci cylindrical, J–, (175–)210–250(–340) × 15–21 µm, with 8 ascospores. Subhymenium dense textura intricata, dark brown, compact. Ascospores uniseriate, broadly ellipsoid, with a single guttule, and smaller guttules at the poles, 19–26.0 × (11–)12–15 µm, Q = 1.68–1.94; wall 1.0–1.5 µm thick, hyaline. Paraphyses slender, hyaline, broadly clavate, 7–10 µm diam. at the tip, 5–6 µm diam. in the middle, septate. Ectal excipulum 75–87(–115) µm thick, dark brown of textura globulosa to textura angularis, cells round to irregular to polygonal, 16–30 × 13–16 µm, walls brown, 1–2 µm thick (especially thicker in the outermost cells), outer cells aggregated to form warts. Medullary excipulum (40–)50–70(–80) µm thick; dense textura intricata, cells appearing angular to irregular to roundish, becoming smaller towards the subhymenium, cells 8.5–20.5 × 5–12 µm. Hairs arising from globose ectal excipular cells, septate and forming a mat of brown hyphae holding soil particles. When young thin, straight, light brown, smooth, (6–)7.5–10.0(–16) µm diam.; with age branched, twisted and curved, dark brown, ≤(40–)50–70(–80) µm diam., with dark granular walls and granular content; hair walls 0.8–1.5(–2) µm thick.

Additional material studied: **PAKISTAN**, **Punjab**, **Lahore**, University of the Punjab, Botanical Garden, 31°29′56″N 74°17′57″E, 16 June 2011, MSM#00160 (LAH 310099!); 5 May 2013, leg. M. Saba, T. Ashraf & A.N. Khalid, MSM#00163 (FH 01142414; GenBank KY805996).

### Molecular analyses of the genus *Geopora*

Initial BLAST analysis of the *G. ahmadii* ITS sequence showed a maximum identity of 84% with collection TAAM 192330 (GenBank accession number FM206472). This collection belongs to clade IX (sensu Tamm & al. 2010) but was identified based on morphology as *G. arenicola* (Lév.) Kers. Our data matrix included 53 isolates, all representing identified *Geopora* species except for three unidentified ectomycorrhizal isolates (El Karkouri & al. 2004, Rincón & al. 2007) and the two outgroup sequences (Table 1). The final aligned data matrix included 722 characters, of which 272 were constant and 351 were parsimony-informative.

Our MP and ML analyses (Fig. 3) largely agree. One exception is the placement of *G. gilkeyae* (Burds.) Guevara & al. (as "*Geopora cooperii* var. *gilkeyi*" in Smith & al. 2007) and *G. tolucana* Guevara & al. In the MP topology (not shown), these species are sister taxa and form a branch basal to all other *Geopora* species (sensu Tamm & al. 2010). However, there is no bootstrap support for this placement. Also, the placement of *G. gilkeyae* in the ML analysis is unresolved (no bootstrap support). In the three analyses (MP, ML, Bayesian), a number of the basal branches lack support, and phylogenetic reconstructions based on ITS alone cannot resolve relationships of *Geopora* at deeper nodes.

Clades I through X (sensu Tamm & al. 2010) are recognized here with high support from MP, ML, and Bayesian analyses (Figs 3, 4). *Geopora ahmadii* is inferred in *Geopora* clade IX from Tamm & al. (2010) with maximum support. The morphological characters of *G. ahmadii* (fruit-body features, ascospore dimensions, Q ascospore values) are consistent with those of clade IX as given by Tamm & al. (2010). The species is recovered as sister to "*Geopora* sp. b" (sensu Schumacher 1979; defined in the study of Tamm & al. 2010) in clade IX. *Geopora* sp. b is known only from northern Europe, with records from Estonia (Tamm & al. 2010) and Norway (Schumacher 1979).

### Discussion

Tamm & al. (2010) reviewed the history of the genus *Geopora* and confirmed that delimitations of species within *Geopora* are difficult. All

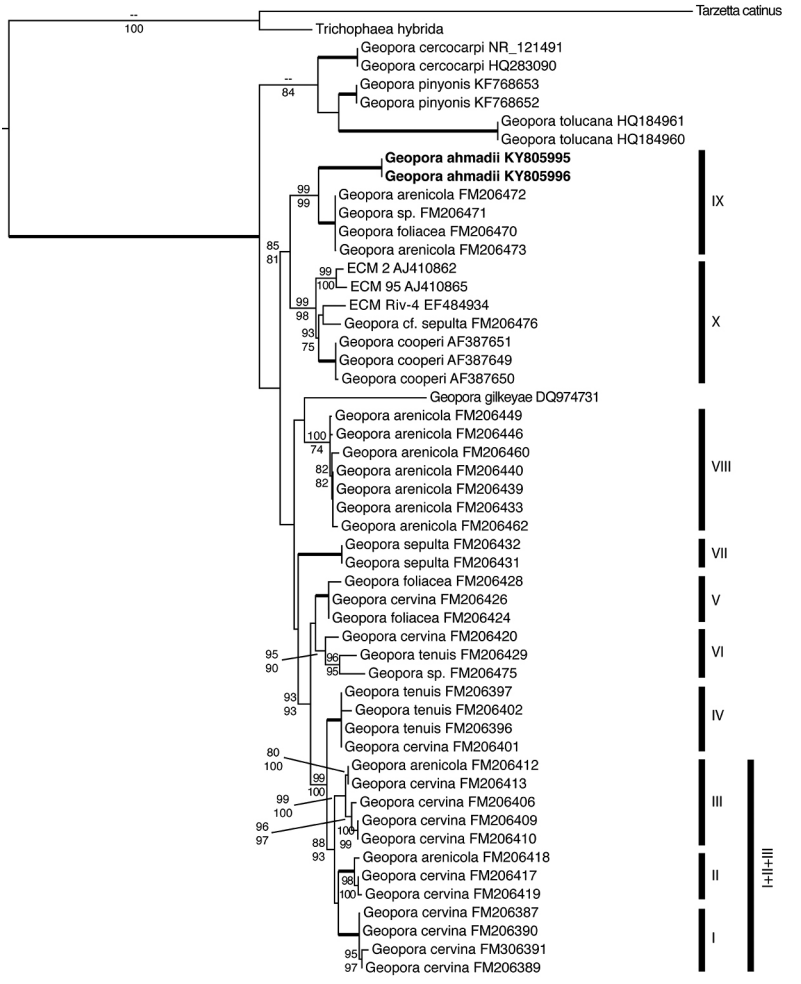


Fig. 3. Phylogeny of *Geopora* species produced from maximum likelihood (ML) analysis of the ITS rDNA dataset. The best-scoring ML tree (log likelihood of –6541.843463) is shown. Only bootstrap values >70% are given. Maximum parsimony (MP) bootstrap values are given above the branches; ML bootstrap values are below the branches. Branches in bold have maximum support.

known taxa are ectomycorrhizal (Tedersoo & al. 2006) but we have not been able to determine the mycorrhizal associate(s) for our new species. The ectomycorrhizal host could be *Pinus roxburghii,* which grows near the site where this species has been repeatedly collected in the Botanical Garden

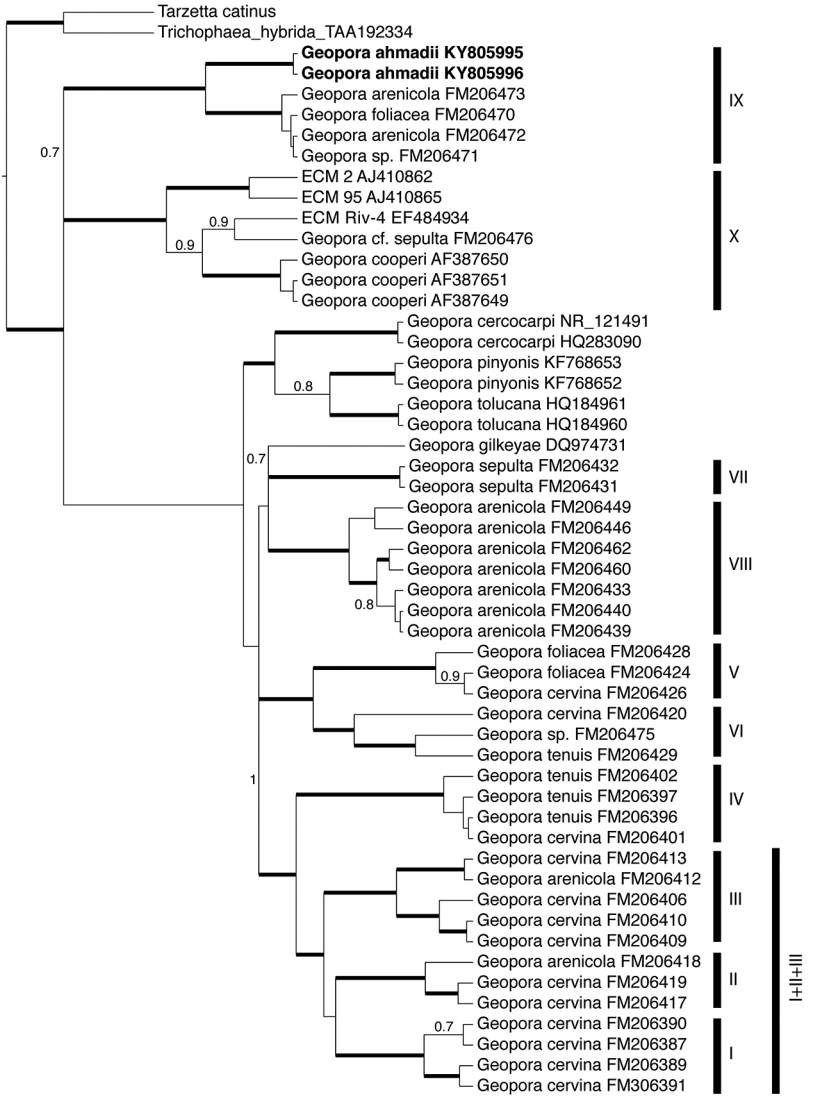


Fig. 4. Phylogeny of *Geopora* species produced from Bayesian inference of the ITS rDNA dataset. Only posterior probabilities ≥0.9 are shown. Branches in bold have maximum support.

of the Department of Botany, University of the Punjab, Lahore, Pakistan. Associations of the new species should be evaluated with sequences from root tip samples.

*Geopora ahmadii* is distinct from other *Geopora* species based on apothecial shape, partial immersion of the apothecium in the soil, hymenial color, and ascospore size. Other partly immersed species are *G. tenuis* (Fuckel) T. Schumach. and *G. cervina* (Velen.) T. Schumach. (Yao & Spooner 1996), which are distinguished by different spore sizes ((20.3–)21.4(–23.0) × (10.8–)11.6(–12.1) for *G. tenuis;* (20.8–)23.8(–26.2) × (10.8–)12.1(–14.2) µm for *G. cervina;* Tamm & al. 2010) and placement in different clades in our analyses. The hymenial color in *G. ahmadii* is similar to *G. sepulta* (Fr.) Korf & Burds., which differs by complete immersion in the soil and placement in another clade.

Our molecular analyses place *G. ahmadii* in clade IX sensu Tamm & al. (2010). Its morphological characters—including the position and shape of the ascomata and ascospore dimensions—are consistent with placement among the other clade IX species. This clade includes specimens initially identified as *G. arenicola* and *G. foliacea* (Schaeff.) S. Ahmad. *Geopora arenicola*, as defined by Tamm & al. (2010), has a completely immersed ascoma. The name *G. foliacea* has been variously applied.

Once again, this study underscores the difficulties of using morphology for species delimitation in this group. Southworth & al. (2011) also noted the difficulty in using morphology to describe their new species, *G. cercocarpi,* citing its exclusive association with *Cercocarpus ledifolius* and that other *Geopora* specimens showed similar host fidelity. Unfortunately we do not know the associate of *G. ahmadii* but we plan to collect root tip samples under trees of its most likely candidate ectomycorrhizal associate, *Pinus roxburghii*.

One comment regarding *G. arenosa* (Fuckel) S. Ahmad: Ahmad (1978) transferred *Peziza arenosa* Fuckel [≡ *Humaria arenosa* (Fuckel) Fuckel] (Fuckel 1864, 1866, 1870) to *Geopora* based on a collection he studied from Rawalakot, Pakistan. According to his description, *G. arenosa* has globose to subglobose apothecia, a whitish gray hymenial surface, and ellipsoid ascospores measuring 20–24 × 13–14.5 µm. Yao & Spooner (1996) studied the type material of *G. arenosa* (Fuckel's Fungi Rhenani exsiccati, No. 1212, K—K(M) 69362, designated as lectotype by Yao & Spooner 2003), and their description contrasts with the material studied by Ahmad (1978) in ascospore shape and size (ellipsoid to fusoid, 27–30 × 13.5–15 µm in the *G. arenosa* type vs. ellipsoid, 20–24 × 13–14.5 µm in Ahmad's material). Because of these discrepancies, we believe that Ahmad's collection from Rawalakot represents a previous collection of our new species. We have not

been able to locate Ahmad's specimen and it is likely lost. It is not present in LAH and no sequence data are available for the Rawalakot material. Further collecting in Rawalakot may help to resolve the identity of Ahmad's species and to determine if it is indeed conspecific with *G. ahmadii*.

**Acknowledgments**

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